Mechanisms of Carcinogenesis by Crystalline Silica in Relation to Oxygen Radicals

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The carcinogenic effects of crystalline silica in rat lungs were extensively demonstrated by many experimental long-term studies, showing a marked predominance for adenocarcinomas originating from alveolar type II cells and associated with areas of pulmonary fibrosis (silicosis). In contrast with its effects in rats, silica did not induce alveolar type II hyperplasia and lung tumors in mice and hamsters, pointing to a critical role for host factors. Using these animal models, we are investigating the role of cytokines and other cellular mediators on the proliferation of alveolar type II cells. Immunohistochemical localization of TGF-β1 precursor in alveolar type II cells adjacent to silicotic granulomas was shown to occur in rats, but not in mice, and hamsters, suggesting a pathogenetic role for this regulatory growth factor. Recent investigations in our laboratory on the biologic mechanisms of crystalline silica included determination of anionic sites on crystalline silica surfaces by binding of the cationic dye Janus Green B; binding of crystalline silica to DNA, demonstrated by infrared spectrometry; production of oxygen radicals by crystalline silica in aqueous media; induction of DNA strand breakage and base oxidation *in vitro* and its potentiation by superoxide dismutase and by hydrogen peroxide; and induction by crystalline silica of neoplastic transformation and chromosomal damage in cells in culture. On the basis of these *in vitro* studies, we propose that DNA binding to crystalline silica surfaces may be important in silica carcinogenesis by anchoring DNA close to sites of oxygen radical production on the silica surface, so that the oxygen radicals are produced within a few Å from their target DNA nucleotides. — Environ Health Perspect 102(Suppl 10): 159–164(1994)

Key words: crystalline silica, quartz, cristobalite, tridymite, silicosis, lung carcinogenesis, DNA binding, DNA damage, Janus Green B, neoplastic transformation, lung, alveolar type II cells, cytokines, TGF-β1

Introduction

In the past decade, the carcinogenic effects of crystalline silica in rat lungs have been extensively demonstrated in as many as 17 experimental groups of rats, including three strains (Fischer 344, Sprague–Dawley, and Wistar) and both sexes, following exposure either by inhalation or by intratracheal instillation to several samples of quartz dust. The tested quartz samples included min-U-sil 5, hydrofluoric acid (HF)-etched min-U-sil 5, DQ12, novaculite (a microcrystalline variety of quartz), and samples of quartz-bearing shale. The results of all these experiments from six different laboratories (*1–8*) have been recently reviewed (*6*). They show that exposure to quartz dusts resulted in the induction of peripheral lung tumors in rats, with a marked predominance for adenocarcinomas of alveolar origin, but also including undifferentiated carcinomas and epidermoid carcinomas (the latter were more frequent in the results from two laboratories).

Experiments in our laboratory (*5,6*), conducted by single intratracheal instillation of quartz (min-U-sil 5 or HF-etched min-U-sil 5) in Fischer 344 rats, examined the development of the pathologic reactions to quartz observed at serial sacrifices from the first day to 6 months, then at 11 and 17 months, and then in rats that died afterwards (17–26 months). The early phases of the lung reaction to quartz included recruitment of both alveolar and interstitial macrophages with uptake and internalization of particles. The next phase, developing from the second week on, showed the formation of nodular granulomas composed of macrophages and fibroblasts, accompanied by focal areas of hyperplasia of the alveolar type II cells. The silicotic granulomas extended progressively in time and the focal hyperplasia of alveolar type II cells became more extensive, sometimes revealing an adenomatoid pattern. Lung tumors were found with increasing frequency at the 11- and 17-month sacrifices. In rats that died between 17 and 26 months, lung tumors were found in 75 to 100% of the rats examined in five quartz treatment groups. We found a higher incidence of lung tumors in females than in males at the 11- and 17-month sacrifices. Females also showed a higher tumor multiplicity and a higher ratio of adenocarcinomas to adenomas than did the males. Rats of both sexes, treated with a single intratracheal instillation of hematite (ferric oxide) dust, for comparison, showed phagocytosis of the dust in macrophages, but they showed no significant fibrosis, no alveolar type II hyperplasia, and no long-term development of lung tumors.

Comparative studies with a similar methodology performed in our laboratory on mice and hamsters showed a remarkable species specificity in the lung pathology induced by a single intratracheal instillation of crystalline silica (*6*). Rats, as stated above, showed a marked fibrogenic reaction, accompanied by hyperplasia of alveolar type II cells and followed by the frequent development of lung tumors. Mice (of three strains) showed moderate fibrosis, but no persistent epithelial hyperplasia and no lung tumor induction. Hamsters (Syrian golden, inbred) showed only macrophagic storage of crystalline silica dust without fibrogenesis and showed no epithelial hyperplasia. The lack of tumor induction by crystalline silica in hamsters had been previously reported (*4*).

The marked species differences in the pulmonary response to crystalline silica

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point to a critical role for host factors. The susceptibility for fibrogenesis may not be determined by the same host factors that influence the carcinogenic response. We hypothesized (*5,6,9*) that the different pathways observed in three rodent species may correspond to differences in host susceptibility to fibrogenesis and to carcinogenesis in different subsets of the human population. Identification of the critical host factors in the three rodent species may lead to an understanding of susceptibility mechanisms in different groups of human subjects.

We decided to investigate the following problems: determination of reactive sites on crystalline silica surface; binding of crystalline silica surface to DNA and/or induction of DNA damage; the role of oxygen radicals in the DNA damage induced by crystalline silica; induction by crystalline silica of neoplastic transformation and/or chromosomal damage; influence of the granulomatous/fibrogenic lung reaction induced by crystalline silica (silicosis) on epithelial proliferation and carcinogenesis.

Detection of Reactive Sites on Crystalline Silica Surface

A newly developed spectrophotometric method measures surface adsorption of the cationic dye Janus Green B to crystalline silica particles in aqueous suspension (*10*). Twelve preparations of crystalline silica were assayed by this method and also for specific surface area by the Brunauer–Emmet–Teller (BET) method (*11*) of surface adsorption of nitrogen gas. Samples tested included 10 preparations of α-quartz: min-U-sil 5, five size-fractionated samples of min-U-sil 10, HF-etched min-U-sil 5, DQ-12, F600, and Chinese standard quartz. Two synthetic preparations of cristobalite and tridymite were also tested.

A strong linear correlation was found between the Janus Green B adsorption method and the BET method of measurement for the twelve samples ($r = 0.977$). All crystalline silica samples tested, including the synthetic preparations cristobalite and tridymite, conformed to the same linear relationship. The correlation was strongest ($r = 0.991$) for the commonly derived size-fractionated min-U-sil samples (Figure 1). Among four standard α-quartz samples tested, min-U-sil 5 and F600 had the lowest specific surface areas, whereas DQ-12 and Chinese standard α-quartz had much higher surface areas. The linear relationship between Janus Green B binding and BET surface area suggests that the ratio of aqueous surface charge to surface area is relatively constant for different crystalline silica preparations.

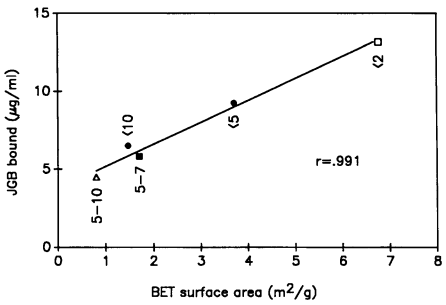

Figure 1. Correlation of Janus Green B binding to specific surface area of size-fractionated min-U-sil (MQZ) quartz. Janus Green B binding was determined after incubation of MQZ (500 mg/ml suspension) with Janus Green B (25 μg/ml in 10 mM phosphate buffer, pH 7.4). Following a centrifugation step, spectrophotometric absorption at 595 nm was determined for the supernatants and the amount of Janus Green B bound per sample was calculated based on a standard curve of Janus Green B absorption versus concentration (*10*).

Binding of Janus Green B to the negatively charged crystalline silica surface is consistent with a charge binding mechanism. Bound dye maintains its color, indicating that it remains in its ionized form. We are currently investigating the correlation of the binding of the cationic dye with surface charge, as determined by zeta potential, which measures mobility of suspended particles subjected to an electric field (*12*).

The polymer poly(2-vinylpyridine-*N*-oxide) (PVPNO) is believed to bind to silanol groups on the crystalline silica surface (*13*). We determined the binding of PVPNO to the same crystalline silica samples listed above, and also found it to be strongly correlated to the surface area as determined by the BET method (*10*). The binding of PVPNO to crystalline silica did not interfere with Janus Green B binding, indicating that they reacted at different ligand sites—silanol groups for PVPNO and ionized groups for Janus Green B, respectively (*10*).

The Janus Green B binding assay represents a useful new technique for the assessment of surface characteristics of crystalline silica samples. Its advantages, compared to the BET method, include improved sensitivity, lack of requirement for specialized instrumentation, and the ability to make rapid simultaneous determinations on multiple samples. Comparison of surface area and surface charge for different preparations of crystalline silica is important in understanding the relative activities of these preparations in studies on mechanisms of silicosis and silica-induced lung cancer.

Crystalline Silica Binding to DNA, DNA Damage and the Role of Oxygen Radicals

The possibility of an interaction between crystalline silica and DNA was investigated by Fourier transform infrared spectroscopy (FT-IR)(*14*). Upon coincubation in aqueous buffer, alterations were observed in both DNA and quartz spectra, suggesting that a DNA–silica complex was formed as quartz interacted with DNA. DNA remained in the B-form conformation in the DNA–silica complex. Following coincubation with quartz in H_2O, the most prominent changes in the DNA spectrum occurred in the 1225 to 1000 cm^{-1} region: the PO_2^- asymmetric stretch at 1225 cm^{-1} was found to be increased in intensity and shifted to lower frequencies; the PO_2^- symmetric stretch at 1086 cm^{-1} was markedly increased in intensity, whereas the band at 1053 cm^{-1}, representing either the phosphodiester or the C—O stretch of the DNA backbone, was significantly reduced in intensity (Figure 2).

When DNA was exposed to increasing concentrations of quartz in D_2O buffer, the DNA spectrum revealed a marked increase in intensity of the peak at 1086 cm^{-1} and a progressive decrease in the intensity of the peak at 1053 cm^{-1}. Additional changes occurred in the area between 1600 and

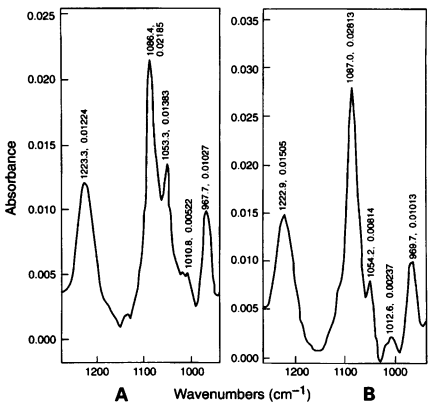

Figure 2. Attenuated total reflectance Fourier transform infrared spectra of DNA and of DNA coincubated with quartz (min-U-sil 5 [MQZ]) in phosphate-buffered H_2O at pH 7.4, in the region 900 to 1300 cm^{-1}. (*A*) DNA alone (10 mg/ml). (*B*) DNA (10 mg/ml) + MQZ (5 mg/ml) – MQZ (5 mg/m) × 1.025. The subtraction factor of 1.025 is derived from the subtraction spectrum of DNA + MQZ – MQZ in D_2O. Note the different absorbance scale in the two panels.

1750 cm^{-1}, related to carbonyl groups of the DNA bases.

The FT-IR spectrum of quartz also showed modifications upon coincubation with DNA: the changes in the quartz spectrum were consistent with an Si–O bond perturbation on the surface of the quartz crystal (*14*). These FT-IR findings, obtained with two samples of quartz (min-U-sil 5 and Chinese standard) that gave similar results, are indicative of an effective hydrogen bonding interaction between surface silanol groups and the phosphate–sugar backbone of DNA.

We suggest that the binding of quartz to DNA is important in the induction of DNA damage due to oxygen radicals. Others have shown that quartz particles in aqueous suspension produce oxygen free radicals, which are detectable by electron spin resonance (ESR) spin-trapping techniques (*15,16*). We recently reported that these quartz-derived radicals can cause damage to DNA *in vitro*, which can be monitored using a simple electrophoretic assay (*17*). Damage to linear DNA was detected as a smearing of discretely sized plasmid DNA bands following incubation with quartz, alone or in the presence of radical modifying agents (H_2O_2, SOD, catalase, deferoxamine, and other metal chelators) (*17*). Thus DNA serves as a sensitive indicator molecule for monitoring free radical production by quartz and the effects of *in vitro* manipulations of free radical pathways.

Production of oxygen free radicals by crystalline silica alone is a continuous process, as shown by the long incubation times necessary to detect DNA strand breakage by the electrophoretic assay. While linear DNA was stable in buffer alone, quartz produced increasing amounts of DNA damage over time, clearly detectable after an incubation period of more than 3 weeks. Five standard quartz preparations and two synthetic preparations of cristobalite and tridymite differed significantly in their ability to mediate *in vitro* DNA strand breakage (Figure 3).

The relative rates of DNA strand breakage by the tested preparations of crystalline silica were found to be directly correlated with the production of the oxidized DNA base, thymine glycol, as measured by gas chromatography–mass spectrometry (data not shown) (*18*).

The effects of modifying agents on DNA strand breakage are consistent with a mechanism of free radical production in which trace quantities of metal, adsorbed to the crystalline silica surface, are involved in catalyzing the production of hydroxyl radicals from H_2O_2 by the Haber–Weiss reaction. The H_2O_2 substrate is derived, at least in part, from the dismutation of superoxide, produced at the crystalline silica surface by the reduction of dissolved molecular oxygen (*17*).

We studied the effects of crystalline silica in cultured cell lines. Quartz particles, <5 μm (min-U-sil 5), were suspended in culture medium and added to cell lines of fetal rat lung epithelial cells (FRLE) or of mouse embryo fibroblastic cells (BALB/3T3/A31-1-1). Both cell lines actively internalized crystalline silica particles, mostly in cytoplasmic phagosomes. Electron microscopic observations showed that several quartz-treated cells contained small (<0.5 μm) particles of quartz in their nuclei, confirmed by energy dispersive X-ray spectroscopy (*19,20*). This finding suggests that a direct interaction of crystalline silica with the genetic material may occur in cells, following silica uptake.

The results so far obtained demonstrate the production of oxygen radicals by crystalline silica surfaces in aqueous buffer, and indicate that they mediate *in vitro* DNA strand breakage and formation of oxidized DNA bases. However, the hydroxyl radi-

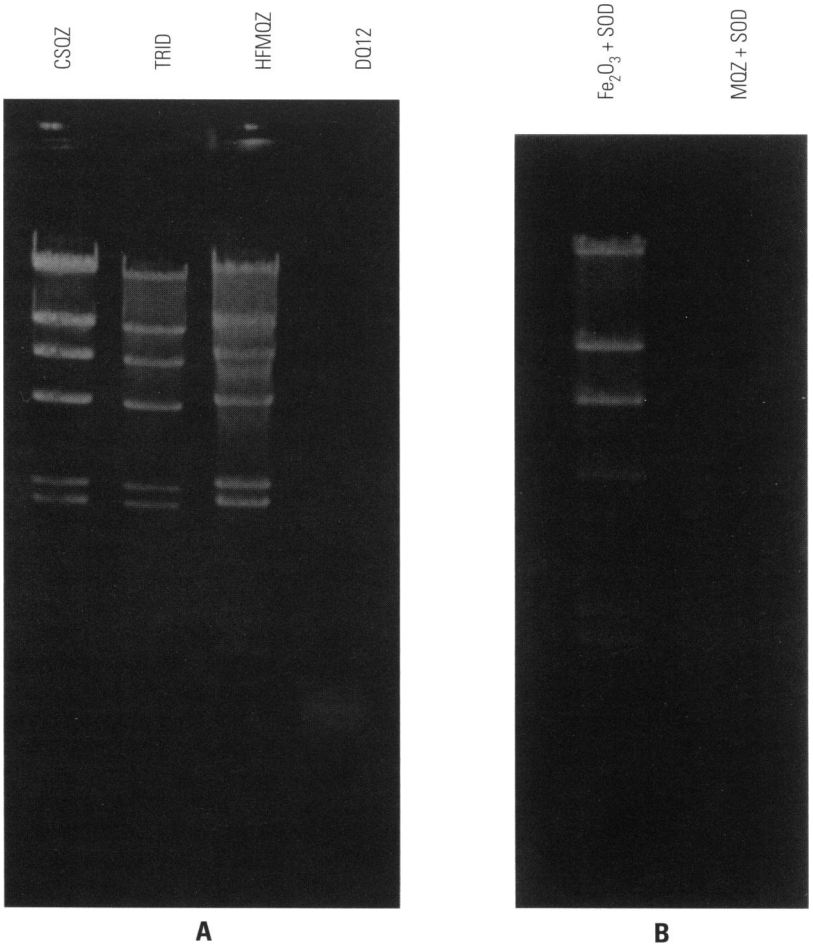

Figure 3. DNA damage by crystalline silica. Samples were incubated with λ Hind III digest DNA in 10 mM phosphate buffer, pH 7.4, at 37°C. One milligram of DNA was removed from the supernatant of a centrifuged reaction at specified time points and electrophoresed in 0.7% agarose (*17*). (*A*) DNA damage by crystalline silica in the presence of additional H_2O_2 (1.5%). At this time point (42 hr), DNA damage by CSQZ was not detectable (confirmed in repeated experiments), damage by tridymite was minimal, damage by HFMQZ was moderate, and damage by DQ-12 was extensive. DNA treated with other silica samples (MQZ, F600, and cristobalite) was completely degraded at 42 hr (not shown). Earlier time points showed damage to be most rapid for the cristobalite sample, followed by F600 and MQZ (not shown). (*B*) Acceleration of DNA damage by SOD. DNA damage by MQZ alone was detectable only after 4 weeks of incubation (data not shown). Manganese SOD (not shown) and CuZn SOD both shortened the time to detectable DNA damage. The control dust, hematite (Fe_2O_3), did not cause DNA damage in the presence of SOD.

cal, which is responsible for most if not all of this DNA damage, has a reaction distance of approximately 15 Å, less than the width of the DNA helix. Thus, in order for this radical to induce a mutagenic effect *in vivo*, silica particles and DNA would have to be very close to each other.

The results obtained by infrared spectrometry, showing that crystalline silica can bind to DNA at the phosphate backbone, suggest a likely mechanism for the effective induction of DNA damage. We propose that the DNA binding to the crystalline silica surface is important in silica carcinogenesis by anchoring DNA close to sites of oxygen radical production on the silica surface, so that the oxygen radicals are produced within a few Å from their target DNA nucleotides.

Neoplastic Transformation and Chromosomal Aberrations Induced by Crystalline Silica

The induction of cytotoxicity and neoplastic transformation by quartz was first reported in Syrian hamster embryo cells (*21*). We studied it in the BALB/3T3/A31-1-1 mouse embryo cell line, which had been previously characterized for transformation assays in our laboratory. Five samples of quartz (min-U-sil 5, HF-etched min-U-sil 5, DQ12, F600, and Chinese Standard Quartz) were tested at final quartz concentrations of 6.5, 12.5, 25, 50, or 100 $\mu g/cm^2$ (*6,20*). All tested quartz samples showed a dose-dependent induction of neoplastic transformation at lower doses, followed by a plateau response at higher doses. Morphologically transformed foci, subcultured and tested for tumorigenicity in nude mice, were all rapidly tumorigenic. Control and transformed cell lines were examined for karyotypes and chromosome abnormalities: all transformed cell lines showed one or more altered chromosomes not seen in the untreated cell line (*6,20*). These findings, to be reported in detail elsewhere, confirm at the cellular level that quartz consistently induced cellular lesions resulting in neoplastic transformation. Further studies are needed to investigate the mutagenic activity of crystalline silica in appropriate cellular systems, such as the human–hamster hybrid A_L cell line, in which a strong mutagenic activity was demonstrated for chrysotile and crocidolite asbestos (*22*). Additional studies, using cell line FRLE (*23*), are in progress in our laboratory (*19*), to investigate the effects of crystalline silica on this appropriate target cell type, which retains the characteristics of alveolar type II cells.

Mesenchymal/Epithelial Interactions and the Role of Cellular Mediators in the Pathogenesis of Silicosis and Associated Lung Carcinogenesis

We have considered the direct interactions of crystalline silica with target DNA and target cells in culture. The lung reactions to crystalline silica *in vivo*, resulting in silicosis and associated lung carcinogenesis, also are dependent on host factors, as demonstrated by the different response patterns in three rodent species, discussed above. Because in rats, the species most susceptible to both fibrogenesis and carcinogenesis, the induction of alveolar type II hyperplasia and lung tumors occurs in close association with silicotic lesions, we have hypothesized (*6,9,24*) that cellular mediators are important in the stimulation of the epithelial proliferative reaction. Such mediators may include various cytokines detected in the silicotic tissues (IL-1, IL-6, TNF-α, TGF-β), possibly mast cell products, as well as oxygen radicals produced by macrophages in the granulomatous reaction. Information on the role of these factors in the different animal models for crystalline silica is still scanty (*6*). Recent studies in our laboratory were devoted to the immunohistochemical localization of TGF-β1, a multifunctional growth factor, in quartz-induced lung lesions in rats (*24*). The results showed that TGF-β1 precursor (indicative of the site of synthesis) was localized in macrophages in the early stages of lung reaction to quartz, and, more prominently after a few weeks from quartz exposure, in the hyperplastic alveolar type II cells. Mature TGF-β1 was localized, intracellularly, in macrophages and fibroblasts at the periphery of silicotic granulomas and in the stroma adjacent to hyperplastic alveolar type II cells, whereas the extracellular mature TGF-β1 was localized in the collagenous stroma adjacent to hyperplastic alveolar type II cells. Interestingly, the cells of adenomas retained a strong localization of TGF-β1 precursor, but those of carcinomas were negative, indicating that a downregulation of TGF-β1 occurs when these cells become malignant. Current studies on the localization of TGF-β1 in the lung of other quartz-treated species indicate that it is minimal in mice and not detected in hamsters, suggesting a critical role for TGF-β1 in the pathogenesis of silicosis-associated lung carcinogenesis. We are also investigating the expression and localization of proteins encoded by selected oncogenes and tumor suppressor genes, to identify the molecular pathways corresponding to this type of carcinogenesis.

Much further work is needed to characterize the critical host control mechanisms and pathways in animal models and in human subjects following exposure to crystalline silica.

Conclusions

The biologic effects of crystalline silica and other inhaled minerals, including both particulates and fibers, have become the focus of increasingly sophisticated investigations. The role of oxygen radicals in the pathogenesis of lung diseases induced by mineral particles and fibers has become evident.

In addition, we suggest that reactive intermediates, generated in nitric oxide (NO) reactions, may also play an important pathogenetic role in these diseases. The mechanisms involving nitrogen radicals are currently being studied in our laboratory (*25*).

As more attention is given to the mechanisms of induction of diseases caused by inhaled minerals, including neoplasia as well as fibrosis, research needs to be addressed to several issues, including the following: mechanisms by which DNA damage by mineral particles occurs in target cells; intracellular site(s) of such interaction, including the question of nuclear penetration of mineral particles and its significance; identification of the radical species generated and the conditions required for their generation and biologic activity in the cellular milieu; the role of scavengers and inhibitors in the pathways leading to specific biologic effects, in relation to cellular and tissue environments; possible differences in free radical mechanisms identifiable in animal species corresponding to different levels of susceptibility for fibrogenesis and carcinogenesis by crystalline silica.

The ultimate challenge is this area of research is to correlate the knowledge acquired through experimental studies—at the tissue, cellular, and molecular level—with the events taking place in the lungs of human subjects, in order to elucidate the mechanisms of lung injury, their inhibition, and their possible prevention.

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